Experimental Rabies in Skunks: Oral, Nasal, Tracheal and Intestinal Exposure

K. M. Charlton and G. A. Casey*

ABSTRACT

Striped skunks (Mephitis mephitis) were exposed to challenge virus standard rabies virus by feeding infected mouse brain in suspension or as intact brain free choice, by forced feeding of suspension, and by intranasal, intratracheal and intraintestinal instillation of suspension. All of five skunks exposed intranasally, two of five exposed intratracheally and two of ten exposed by forced feeding developed rabies. None of the skunks exposed to challenge virus standard virus, by other methods, became rabid. Most of the survivors, when challenged intramuscularly with street rabies virus at six months, developed rabies. The results indicate that the skunk is much more susceptible to challenge virus standard rabies virus given intranasally than by the other methods used. When disease occurs following oral administration, infection may be associated with prolonged contact with buccal mucosa or accidental contact with nasal mucosa. Survivors had little or no protection when challenged intramuscularly with street rabies virus.

RÉSUMÉ

Cette expérience visait à infecter des moufettes (Mephitis mephitis) avec la souche du virus rabique fixe "CVS", à l'aide des méthodes suivantes: libre accès à des cerveaux de souris rabiques, intacts ou en suspension, alimentation forcée ou instillation intra-nasale, intra-trachéale et intra-intestinale avec une suspension virulente de cerveaux de souris. La rage se développa chez les cinq moufettes inoculées par la voie intra-nasale, chez deux des cinq inoculées par la voie intra-trachéale et chez deux des dix qu'on avait soumises à l'alimentation forcée. Aucun cas de rage ne se développa chez les moufettes inoculées par les autres méthodes expérimentales. La plupart des survivantes développèrent la rage à la suite de l'injection intra-musculaire d'une souche du virus de rues qu'on leur administra, six mois plus tard; elles ne possédaient que peu ou pas de protection, lors de cette injection.

Les résultats de cette expérience révèlent que la moufette s'avéra beaucoup plus vulnérable à l'injection de la souche "CVS" par la voie intra-nasale qu'autrement. Les cas de rage consécutifs à l'administration orale de matériel virulent s'expliqueraient par un contact prolongé du virus avec la muqueuse buccale ou par un contact accidentel avec la muqueuse nasale.

INTRODUCTION

In nature, rabies is transmitted mainly by transfer of infective saliva by biting rabid animals. However, there is some evidence that oral and nasal infections occasionally contribute to spread of the disease (1, 8, 12). Experimentally, foxes (6, 11) are fairly susceptible to rabies virus given orally but skunks do not consistently develop rabies when exposed by this method (4, 15). Similarly foxes can be readily immunized by rabies vaccine virus given into the mouth (2, 3, 5, 10, 13) or directly into the intestine (by enteric coated sugar non-pareils), but preliminary studies suggest that the skunk is more refractory than the fox to immunization via these routes (Dr. J.G. Debbie, personal communication). Observations concerning the susceptibility of skunks to virulent virus administered by various nonparenteral routes may be of value in determining

*Animal Pathology Division, Health of Animals Branch, Agriculture Canada, Animal Diseases Research Institute (E), P.O. Box 11300, Station H, Ottawa, Ontario K2H 8P9.

Submitted October 20, 1978.

the most efficacious route to use for vaccination in the field.

MATERIALS AND METHODS

Virus

Weanling, white, Swiss mice were inoculated intracerebrally with challenge virus standard (CVS) rabies virus and brains were harvested when the mice were moribund. Whole, infected mouse brains were used in some trials and 10% suspension of brain in diluent (0.1M phosphate buffer, pH 7.4 with 2% inactivated horse serum) was used in others. Titrations were made in weanling mice to determine the mouse intracerebral lethal dose 50 ($MICLD_{50}$) /0.03 ml.

Virus suspension, for intramuscular challenge of survivors, was prepared from salivary glands of naturally infected skunks by homogenization of the glands with diluent (0.1M phosphate buffer, pH 7.4 containing 20% inactivated horse serum, 1000 IU penicillin and 2 mg streptomycin/ml) and centrifugation at 600 g for 15 min. The titer was $10^{6.5}$ $MICLD_{50}$/ 0.03 ml.

Experimental Animals

Male striped skunks (*Mephitis mephitis*), reared in captivity, were purchased from a supplier[1]. They were kept in stainless steel cages and given food and water *ad lib*. The skunks were six to nine months old at the beginning of the experiment.

Experimental Procedure

The principals were divided into seven groups of five based on method of exposure and there was one control group (Table I). Each skunk in group 1 received 90-100 infected mouse brains free choice and those in group 2 were given 10% suspension free choice in water bottles. Each skunk in group 1 consumed approximately 30 g of infected mouse brain ($\sim 10^{8.9}$ $MICLD_{50\ s}$); one-half of the total was given on each of two successive days. Each skunk in group 2 drank approximately 2 ml of suspension. Skunks in group 3 were given 0.5 ml and skunks in group 4 were given 1.0 ml of 10% brain suspension into the mouth by means of a syringe and attached polyethylene tube[2], 3 cm in length. The skunks in group 4 were trained daily for two weeks prior to infection by giving normal mouse brain suspension. This was done to facilitate acceptance and immediate swallowing of positive suspension when given later. Skunks in groups 5-7 were anesthetized with Ketaset[3] (10 mg/lb) and Atravet[4] (0.25 mg/lb) given intramuscularly. Those in group 5 received 1.0 ml of suspension intranasally, those in group 6 were given 1.0 ml intratracheally and those in group 7 were given 5.0 ml into the intestine. For intestinal administration, two polyethylene tubes[5] (No. 7435 tube within No. 7460 tube) were passed through the esophagus and into the duodenum to a point 30 cm distal to the pylorus. The tubes were guided into the duodenum via manipulation through a midline laparotomy. The inner tube was then pushed 10 cm beyond the tip of the outer tube. Suspension was injected through the inner tube which was then withdrawn before removing the outer tube.

Blood for mouse serum neutralization tests (16) was collected before exposure and at one to two months and six months postexposure. The survivors in groups 2-7 were challenged with street rabies virus isolated from skunk salivary glands. In each challenged skunk, the abductor digiti quinti muscle of the right pelvic limb was inoculated with 0.3 ml of 10% suspension of salivary glands (titer $10^{6.5}$ $MICLD_{50}$/ 0.03 ml) from naturally infected skunks. Skunks that developed rabies following exposure to CVS virus or following challenge with street virus were necropsied and parts of the brain were frozen in liquid nitrogen. Both right and left abductor digiti quinti muscles and other selected muscles from several challenged skunks were frozen in liquid nitrogen. Later these tissues were sectioned and examined by the fluorescent antibody technique for rabies virus. Conjugate for this

[1]Ruby's Fur Farm, New Sharon, Iowa.

[2]No. PX046 Medical Grade Polyethylene Tubing. Becton, Dickinson and Co., Rutherford, New Jersey.

[3]Ketamine hydrochloride, Rogar/STB, London, Ontario.

[4]Acepromazinemaleate, Ayerst Laboratories, Montreal, Quebec.

[5]Intramedic polyethylene tubing. Clay Adams, Division of Becton, Dickinson and Co., Parsippany, New York.

TABLE I. Experimental Rabies in Skunks. Oral, Nasal, Tracheal and Intestinal Exposure to CVS Virus

Group	Titer of CVS Virus ($MICLD_{50}$/0.03 ml)	Dose	Route	D/E[a]	Challenge of Survivors with Street Virus D/C[b]
1	$10^{5.9}$	100 infected mouse brains	Orally free choice	0/5	Not done
2	$10^{7.5}$	Free choice in water bottle: Ave. dose 2 ml	Orally free choice	0/5	5/5
3	$10^{7.5}$	0.5 ml of 10% suspension	Orally (syringe & tube)	2/5	2/3
4	$10^{7.5}$	1.0 ml of 10% suspension	Orally (syringe & tube)	0/4	3/4
5	$10^{7.5}$	1.0 ml of 10% suspension	Intratracheal	2/5	3/3
6	$10^{7.5}$	1.0 ml of 10% suspension	Intranasal	5/5	
7	$10^{7.5}$	5 ml of 10% suspension	Intraduodenal	0/5	5/5
8	No dose control	—	—	NA[c]	5/5

[a]Deaths/Exposures
[b]Deaths/Challenged
[c]Not applicable

technique was prepared by labelling serum antibodies from hyperimmunized hamsters with fluorescein isothiocyanate (9).

RESULTS

All of five skunks exposed intranasally, two of five exposed intratracheally and two of five that were force fed (without training) developed rabies (Table I). The incubation period was seven to nine days for all except one skunk exposed intranasally in which signs began on day 12. Usually, there was initial listlessness and ataxia followed soon by increased salivation, paralysis, and occasionally tremors. Each affected skunk was killed or died within two days of onset of clinical signs.

None of the skunks that were fed infected mouse brain or suspension free choice, force fed (with previous training) or given suspension directly into the intestine developed rabies (Table I). One of the skunks force fed suspension (with previous training) died on day 10 but was rabies negative on both the fluorescent antibody and mouse inoculation tests.

One skunk from group 3 had a serum neutralization titer of 1:11 at one month postexposure but was negative at 1:5 dilution at six months. All of the other survivors of the exposure to CVS virus were negative at 1:5 dilution or greater in every test. Most of the survivors succumbed to challenge at six months (Table I). This included the skunk with a titer of 1:11 at one month. All five controls succumbed to challenge.

By the immunofluorescent technique, skunks that developed rabies following exposure to CVS virus had fairly extensive accumulations of antigen in neurons in all parts of the brain, spinal cord and trigeminal ganglia. There was no greater accumulation of antigen in the olfactory lobes than in other grey areas of the central nervous system (CNS). Antigen was not detected, by the fluorescent antibody technique, in salivary glands of skunks infected with CVS virus.

Immunofluorescence occurred in a few extrafusal and intrafusal muscle fibers at the inoculation site of several of the challenged skunks (Table II). The number of affected extrafusal fibers rarely exceeded

TABLE II. Immunofluorescence in Skeletal Muscle of Skunks that Succumbed to Challenge

Group and Skunk	Days between Challenge and Death	RADQ[a]	LADQ[b]	Other Muscles
2 - 1	28	–[c]	IF[d] +	Abductor pollicus brevis: IF +
2 - 2	28	IF +	–	Gluteal: IF +
2 - 3	23	EF[e] +	–	Gastrocnemius: IF +
2 - 5	21	EF +	IF +	—
3 - 3	28	–	–	–
3 - 3	25	–	IF +	–
4 - 1	20	EF + & IF +	–	Abductor pollicus brevis: IF +
4 - 2	37	EF +	–	0
4 - 3	20	IF +	IF +	0
5 - 2	17	EF +	—	0
5 - 3	17	EF +	–	0
5 - 5	27	IF +	IF +	0
7 - 2	23	EF +	–	–
7 - 3	17	–	IF +	0
7 - 5	160	IF +	IF +	Long digital extensor: IF + Gluteal: IF +
8 - 3	17	EF +	IF +	0
8 - 4	16	EF + & IF +	–	0
8 - 5	16	EF +	IF +	0

[a]Right abductor digiti quinti — the inoculation site
[b]Left abductor digiti quinti
[c]0 = not tested; – = no antigen detected in muscle fibers, + = one or more muscle fibers contained antigen
[d]Intrafusal fibers
[e]Extrafusal fibers

30 and these fibers occurred predominantly as solitary fibers or in groups of two to four within one general region of the muscle. Affected fibers contained small granules and occasionally large bodies of antigen distributed throughout most or all of the cross-sectional area. In longitudinal sections the antigen frequently was in linear arrays aligned with the myofibrils and extended for 500-600 μm along the fiber. Affected extrafusal fibers occurred only at the inoculation site, but antigen occurred in intrafusal fibers both at the inoculation site and in other muscles. When muscle spindles were affected, usually all the intrafusal fibers of that spindle contained antigen.

DISCUSSION

Our results demonstrate that, by the methods used, skunks were much more susceptible to CVS rabies virus administered intranasally than when given orally free choice or directly into the intestine. This is in agreement with our previous studies in mice in which infective material (brain or suspension) given free choice rarely caused rabies (7). In other studies, skunks developed rabies after they were fed whole mice infected with CVS rabies virus or a bat strain of street rabies virus (4, 15). In our study, the average dose received by skunks given infected mouse brain or suspension free choice was slightly greater than that received by skunks exposed intranasally. Variations in the dose received probably would not account for the absence of deaths due to rabies in groups given virus free choice. Two of five skunks given suspension by forced feeding (without previous training) became rabid. Perhaps accidental exposure of nasal mucosa or longer exposure of the oral mucosa may be factors in some cases of infection following oral administration in skunks. Also, virus strain may be sig-

nificant, since in one study skunks fed mice infected with an isolate from a silver-haired bat (*Lasionycterus noctivagans*) developed rabies but not skunks given other isolates (4).

The role of nonparenteral routes in transmission of rabies in nature is unknown. The high rate of intranasal infection with CVS virus suggests that aerosol infection with street virus may be possible under some conditions as has been reported with other species (8). It has been suggested that aerosol infection may be a mechanism of transmission in communal denning carnivores, especially skunks (17). According to our results, infection following ingestion of infective material would be unlikely to occur through intact buccal mucosa.

Currently there is considerable interest in wildlife rabies vaccination by the enteric route, i.e. by methods which release vaccine virus into the intestine without prior inactivation in the stomach. In our study, neither infection with disease nor immunity occurred when CVS virus was administered directly into the intestine. Although there has been some success in skunks using vaccine virus by the enteric route (Dr. J.G. Debbie, personal communication), the proportion of vaccinated animals that develop SN titers is probably inadequate for successful use in the field. Possibly refinements in the technique of enteric coating, the use of special vehicles to facilitate absorption, or other vaccine strains at very high dosage will increase the rate of immunization by this method.

Only two of seven skunks previously exposed to CVS virus via oral administration survived challenge with street virus. We do not know whether this was due to immunity (undetected by SN test) or inadequate challenge. Although, in this experiment, all the controls succumbed to challenge, probably 100% mortality would not occur in all trials.

In this present study, antigen occurred in extrafusal muscle fibers at the inoculation site and in intrafusal fibers both at the inoculation site and in other muscles. In a recent study of the pathogenesis, antigen occurred in extrafusal muscle fibers at the inoculation site before it occurred in neurons of the CNS. Terminally intrafusal fibers and rarely extrafusal fibers in muscle remote from the inoculation site contained antigen[6]. These features suggest that extrafusal fibers at the inoculation site were infected directly by virus in the inoculum and that infection of myocytes in other muscles occurred late in the disease as a result of centrifugal migration of virus from the CNS. Hamsters inoculated intramuscularly developed immunofluorescence in muscle fibers before the CNS or dorsal root ganglia, suggesting that replication in myocytes may be involved in transfer of virus to peripheral nerves (14).

ADDENDUM

[6]Charlton, K.M. and G.A. Casey. Experimental rabies in skunks. Immunofluorescent, light and electron microscopic studies. Lab. Invest. In Press. 1979.

REFERENCES

1. **BABES, V.** Traite de la Rage. Paris: Baillière et Fils. 1912. Cited by Winkler, W.G. Airborne rabies. In The Natural History of Rabies. G.M. Baer, Editor. Volume 2. pp. 115-121. New York: Academic Press. 1975.
2. **BAER, G.M.** Wildlife vaccination. In The Natural History of Rabies. G.M. Baer, Editor. Volume 2. pp. 261-266. New York: Academic Press. 1975.
3. **BAER, G.M., M.K. ABELSETH and J.G. DEBBIE.** Oral vaccination of foxes against rabies. Am. J. Epidem. 93: 487-490. 1971.
4. **BELL, J.F. and G.J. MOORE.** Susceptibility of carnivora to rabies virus administered orally. Am. J. Epidem. 93: 176-182. 1971.
5. **BLACK, J.G. and K.F. LAWSON.** Sylvatic rabies studies in the silver fox (**Vulpes vulpes**). Susceptibility and immune response. Can. J. comp. Med. 34: 309-311. 1970.
6. **BLACK, J.G. and K.F. LAWSON.** Further studies of sylvatic rabies in the fox. (**Vulpes vulpes**). Vaccination by the oral route. Can. vet. J. 14: 206-211. 1973.
7. **CHARLTON, K.M. and G.A. CASEY.** Experimental oral and nasal transmission of rabies virus in mice. Can. J. comp. Med. 43: 10-15. 1979.
8. **CONSTANTINE, D.G.** Rabies transmission by nonbite route. Publ. Hlth. Rep., Wash. 77: 287-289. 1962.
9. **DEAN, D.J. and M.K. ABELSETH.** The fluorescent antibody test. In Laboratory Techniques in Rabies. 3rd Edition. M.M. Kaplan and H. Koprowski. Eds. pp. 73-84. Geneva: World Health Organization. 1973.
10. **DEBBIE, J.G., M.K. ABELSETH and G.M. BAER.** The use of commercially available vaccines for the oral vaccination of foxes against rabies. Am. J. Epidem. 96: 231-235. 1972.
11. **KOVALEV, N.A., V.A. SEDOV and A.S. SASHENKO.** Experimental study of some ways in which rabies is transferred. Proc. 19th Wld Vet. Congr. Mexico City. 2: 712. 1971.
12. **MANSELL, G.A.** Dog disease in Northern Quebec. R.C.M.P. Rep. "G" Division. File 49-G, 1974-4-2-MI. Port Harrison. Mar. 10, 1951.
13. **MAYR, A., H. KRAFT, O. JAEGER and H. HAACKE.** Oral Immunisierung von Fuchsen gegen Tollwut. Zentbl. VetMed. B. 19: 615-625. 1972.
14. **MURPHY, F.A., S.P. BAUER, A.K. HARRISON and W.C. WINN.** Comparative pathogenesis of rabies and rabies-like viruses. Viral infection and transit from inoculation site to the central nervous system. Lab. Invest. 28: 361-376. 1973.
15. **RAMSDEN, R.O. and D.H. JOHNSTON.** Studies on the oral infectivity of rabies virus in carnivora. J. Wildl. Dis. 11: 318-324. 1975.
16. **THOMAS, J.B.** The serum neutralization, indirect fluorescent antibody, and rapid fluorescent focus inhibition tests. In The Natural History of Rabies. G.M. Baer, Editor. Volume 1. pp. 417-433. New York: Academic Press. 1975.
17. **WINKLER, W.G.** Airborne rabies. In The Natural History of Rabies. G.M. Baer, Editor. Volume 2. pp. 115-121. New York: Academic Press. 1975.